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Research Article

The potential role of wound-activated volatile release in the chemical defence of the brown alga *Dictyota dichotoma*: Blend recognition by marine herbivores

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Abstract. The chemical defence potential against herbivores of certain Dictyotalean brown algae increases after tissue disruption. This wound activated defence has been explored in bioassays, but the metabolic pathways behind it are unknown. Here we describe a metabolic profiling approach to identify the activated defence metabolites. Before and after tissue damage of *Dictyota dichotoma* modified diterpenes, non-volatile medium polar metabolites as well as volatile compounds were profiled. While comparison of extracted intact and mechanically wounded algae revealed no significant differences in structure and distribution of semi-volatile and reversed phase LC/MS detectable metabolites, a strong release of gaseous volatiles was observed. Solid phase micro extraction (SPME) and GC/MS were used for identification and quantification of these biogenic gases. This showed that *D. dichotoma* released elevated amounts of trimethylamine (TMA) and dimethylsulphide (DMS) after mechanical tissue damage. To study the ecological significance of compounds released post injury and of the biosynthetically connected non-volatile acrylate, choice assays were performed with the amphipod *Amphithoe longimana.* Behavioural assays on artificial diets did not reveal any repellent role for the single isolated metabolites. In strong contrast, a mixture of TMA, DMS and acrylate significantly reduced the association of the herbivores with the treated food pellets. This shows that mixtures of these biogenic gases and acrylate are recognized by the herbivores and influence food selection.

Key words. Dictyotales; dimethylsulphide; trimethylamine; activated defence; mass spectrometry; solid phase micro extraction.

Introduction

Despite large differences between habitats of seaweeds and land plants, both suffer similar constraints such as competition for space, nutrients and light. Also costs caused by pathogens and herbivores are comparable (Hay and Steinberg, 1992). Both, higher

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plants and seaweeds have developed efficient chemical defence systems to overcome these limitations (Hay and Steinberg, 1992; Berenbaum and Rosenthal, 1992; Paul and Puglisi, 2004; Paul et al., 2006). Chemical deterrents are widespread among seaweeds and have important direct and indirect effects on seaweed-herbivore interactions and on benthic community structure in general (Hay and Steinberg, 1992; Paul and Puglisi, 2004). Identified metabolites from marine macroalgae are numerous and many have been demonstrated to deter consumers or to suppress pathogens or fouling organisms (Hay, 1996; Hay and Fenical, 1988; Paul and Puglisi, 2004; Weinberger et al., 2002). However, the potential for chemical defence is also found beyond constitutively produced or stored metabolites: induced chemical defence, a strategy where defensive metabolites are produced *de novo* and on demand, and activated chemical defence, that involves reactive metabolites that are rapidly released from storage metabolites after tissue disruption, can play key roles as well (Cetrulo and Hay, 2000; Cronin and Hay, 1996a; Paul and Van Alstyne, 1992; Pavia and Toth, 2000; Pohnert, 2004; Van Alstyne, 1988).

Biogenic gases such as non–methane hydrocarbons, organohalogens, ammonia, methylamines and dimethylsulphide are produced by many marine micro– and macroalgae (Carpenter et al., 2000; Broadgate et al., 2004; Kwint and Kramer, 1995; Steiner and Hartmann, 1968; Steinke et al., 2002a; Wolfe et al., 1997) and some of these gases have been found to be involved in chemical defence reactions (Pohnert et al., 2007; Van Alstyne and Houser, 2003). One of the best studied gases in this context is dimethylsulphide (DMS). As an important volatile compound from marine organisms it is recognized to be one of the most dominant natural sources of atmospheric sulphur (Andreae and Barnard, 1984). DMS and acrylate are primarily produced upon dimethylsulphoniopropionate cleavage by the enzyme DMSP–lyase (Challenger, 1951; Kiene, 1990; Steinke and Kirst, 1996) or in a base mediated reaction (Kiene, 1990).

DMSP is produced by macroalgae and phytoplankton to control internal osmotic pressure and to serve as a cryoprotectant (Reed, 1983; Dickson and Kirst, 1987; Nishiguchi and Somero, 1992; Andreae and Barnard, 1984; Kwint and Kramer, 1995), although this latter role is unlikely for warm water species. DMS release also plays a role in chemical defence because the DMS produced during herbivore activity can deter further feeding on both micro- and macroalgae (Kiene, 1990; Steinke et al., 2002a; Van Alstyne and Houser, 2003; Van Alstyne et al., 2001; Wolfe et al., 1997).

Other biogenic gases previously detected from micro- and macroalgae are simple volatile amines. These can function as osmolytes (Hartmann and Auferman, 1973; Herrmann and Jüttner, 1977; Steiner and Hartmann, 1968; Steinke et al., 2002b) and it is postulated that they could play a role in chemical defence as well (Gibb et al., 1999; Hartmann and Auferman, 1973).

Bioassays indicated that an activated chemical defence was common among the Dictyotalean brown algae (Cetrulo and Hay, 2000) but the chemical mechanisms underlying this changed susceptibility to consumers has not been investigated. In this study the chemical processes initiated upon tissue disruption of *Dictyota dichotoma* and their potency as a chemical defence are explored. Metabolic profiling of intact and wounded algal tissue pointed towards biogenic gases as active components. Therefore we used a fast solid phase micro extraction (SPME) / gas chromatography/mass spectroscopy (GC/MS) based method to determine the wound activated volatile production. The role of the identified trace gases in the chemical defence was addressed by performing choice assays with a model herbivore, the amphipod *Amphithoe longimana.*

Material and methods

Reagents and culture medium

Analytical grade solvents (Merck, Darmstadt, Germany) were used for extraction and HPLC/MS analysis. Trimethylamine*HCl, acrylic acid and dimethylsulphide (DMS) (Fluka / Aldrich, Seelze, Germany) served as standards for quantification and identification and were used for bioassays. Algae and amphipods were isolated in the field and subsequently kept in artificial seawater medium, prepared by dissolving 33 g / L Instant Ocean (Aquarium Systems, Sarrebourg, France) in distilled water. Agar (BioChemika for microbiology) for the generation of artificial food was obtained from Fluka / Aldrich, Seelze, Germany.

Metabolic profiling

For metabolic profiling of the wound response, weighed pieces (200–500 mg) of *D. dichotoma* were either wounded by grinding in a mortar before extraction (wounded treatment) or shock frozen with excess liquid nitrogen to suppress the action of enzymes and then extracted (control). If wound reactions were monitored for more than 1 h controls were performed with intact algae that were boiled in water to inactivate enzyme activity. Wound reactions were monitored for different time periods (5 min up to

48 hours). Extraction was performed at room temperature in clear glass vials using twice the volume of the algal wet weight of methanol, methanol / water 1:1, dichloromethane, chloroform or following the protocol of Bligh and Dyer (1959).

Profiling of secondary metabolites in chloroform and dichloromethane extracts was performed using a Finnigan TraceMS coupled to a Finnigan ITS gas chromatograph (Thermo–Finnigan, Waltham, MA, USA) equipped with an Alltech EC5 capillary column (15 m x 0.25 mm, 0.25 µm, Alltech, Hamburg, Germany). Methanol and methanol / water extracts were profiled using a Finnigan LCQ with APCI and ESI ion source connected to a Hewlett Packard 1100 System equipped with a Grom–Sil 120 ODS–3 CP column (125 mm x 2 mm, particle size 3 µm, Grom, Rottenburg-Hailfingen, Germany).

Extraction and detection of biogenic gases

For adsorption, a manual solid phase micro extraction fibre holder with a bonded Carboxen™/Polydimethylsiloxane (CAR/PDMS) fibre was used (Supelco™, Deisenhofen, Germany). Algal pieces (200–500 mg) were placed in 4 ml vials that were sealed with a teflon septum. The fibre was inserted in the gas phase over the algal sample for 20 min to collect volatiles and then directly inserted into the injection port of a gas chromatograph for desorption.

Gas chromatography / mass spectrometry was performed on a Finnigan TraceMS coupled to a Finnigan ITS gas chromatograph (Thermo-Finnigan, Waltham, MA, USA) equipped with an Alltech EC5 capillary column (15 m x 0.25 mm, 0.25 µm, Alltech, Hamburg, Germany). The inlet temperature was maintained at 250 °C and samples were injected in splitless mode. The column oven was held at 40 °C for 4 min, programmed from 40 °C to 100 °C at 10 °C / min and from 100 °C to 250 °C at 30 °C / min and finally held at this temperature for 2 min. Helium was used as carrier gas at a constant flow of 1.5 ml / min and the transfer capillary was held at 270 °C.

The high resolution mass measurements were performed on a GCT Premier (Waters Micromass, Manchester, England) connected to an Agilent 6890N gas chromatograph (Agilent, Basel, Switzerland) equipped with an J&W DB5ms column (30 m x 0.25 mm, 0.25 µm) using the same temperature program as above.

Quantification and calibration curves

Calibration curves were determined for TMA between 1 µg TMA*HCl ml^{-1} and 40 µg TMA ml^{-1} artificial seawater medium, and for DMS between 0.5 µg and 12.5 µg DMS ml^{-1} medium in triplicates. For quantification stock solutions of TMA*HCl (10 mg ml^{-1} H_2O) and DMS (10 mg ml^{-1} MeOH) were used. These stock solutions were freshly prepared every day. To reach the required concentrations appropriate amounts of these stock solutions were added to 800 µl medium in 4 ml glass vials, which were sealed immediately with a Teflon lined screw cap. The SPME fibre was directly inserted into the gas phase through the septum. Extraction was carried out for 20 minutes. GC/MS measurements were performed as described above. The calibration curves were obtained by plotting the area ratios of each molecular ion of the analyte relative to the initial applied concentration of these volatiles. A simultaneous detection, even with overlapping signals, of TMA and DMS was possible because of known analytical ions of TMA (30, 42 and the ratio between 58 and 59 *m/z*) and DMS (35, 47 and 62 *m/z*). Additional high resolution mass measurements supported the identification of these ions. Peak areas of verified signals were monitored with single ion traces for TMA at *m/z* 59 and for DMS at *m/z* 62 for quantification.

Sample preparation

To determine the amount of TMA and DMS released upon disruption of algal tissue about 200 mg of *D. dichotoma* or *D. menstrualis* were weighted in 4 ml GC vials pre–equipped with 5–7 glass beads with a diameter of 2–3 mm and 200 µl of culture medium. After sealing with a Teflon lined screw cap the samples were vortexed for one minute. This resulted in homogenisation of ca. 50 % of the thallus material. Subsequently the SPME fibre was introduced for 20 min into the headspace over the wounded algae. In independent experiments it was verified that vortexing in the presence of glass beads did not affect the recovery of the analytes.

Organisms

D. dichotoma was collected from the Atlantic coast in Roscoff, France and transferred to the laboratory in Lausanne, Switzerland within two days. Algae were kept at 16 °C under a 14:10 h light dark regime in artificial seawater medium prepared as described above. Axenic cultures of *D. dichotoma* were provided by the Inselstation Helgoland of the Alfred-Wegener Institute, Germany. *Dictyota menstrualis* and the amphipod *Amphithoe longimana* were collected in Morehead City, North Carolina (USA) and air-shipped to the laboratory, where they were kept in artificial seawater under identical culture conditions as *D. dichotoma.*

Preparation of artificial agar based food

Artificial food was prepared using freeze–dried and finely powdered *Ulva spp.* Therefore 0.72 g of agar

was mixed with 20 ml of distilled water and dissolved by heating in a microwave. Subsequently 2 g of powdered *Ulva* was added to 16 ml of distilled water at room temperature, this was added to the heated agar mixture and vigorously homogenized. This mixture was used to fill 15 ml plastic centrifuge tubes (Falcon, Franklin Lakes, New York, USA). The tubes were inverted and cooled to room temperature. TMA, DMS and / or acrylic acid were syringe injected into the small volume of air enclosed at the bottom of the tube. The closed Falcon tube was heated in a water bath until the agar liquefied followed by brief shaking to homogenize the mixture. After solidification of the agar the tube was opened with a saw and the agar roll removed. This roll with a diameter of 1.5 cm was cut to give ca. 2 mm thick agar discs, which were further cut into four pieces. Control food was prepared using the same protocol, but omitting addition of the volatile components.

Volatile content of the artificial food was monitored before and after incubations. Therefore pieces of TMA and DMS loaded agar prepared as described above were either extracted directly or transferred into 20 ml medium for 25 min. These pellets were transferred into a 4 ml GC vials, sealed airtight and heated in a water bath until the agar liquefied. The determination of volatile compounds was performed as described above giving the effective concentrations of the gases before and after the assay.

Choice assays

Responses of *A. longimana* to agar–based food with and without added volatiles were monitored in choice assays. A concentration range covering three orders of magnitude was adopted. Assays with 39.2 μg DMS and/or acrylic acid g^{-1} and 294 μg TMA g^{-1} added to the agar before mixing, 10x and 100x of this concentration were performed. Feeding assays were performed in 12 well ice cube containers, each well filled with 20 ml of medium (18 °C). One amphipod per vessel could choose between control and treated food pellets that were arranged on specified sides of the assay container. Every five minutes for twenty five minutes, we noted which food each amphipod was on. The same amphipods were used for replications after re-conditioning overnight in untreated medium.

Results and discussion

Previous bioassays indicated that chemical defences of Dictyotalean brown algae are activated by mechanical tissue damage that should simulate many types of herbivore damage (Cetrulo and Hay, 2000). We investigated whether production of volatiles could form the molecular basis of this defence reaction. Comparison of the metabolic profiles of dichloromethane extracts from intact and mechanically wounded *Dictyota dichotoma* using GC/MS indicated that both damaged and control plants contained diterpenes that were similar in structure and distribution (Fig. 1). Changes in these well-studied chemical defences were thus unlikely causes for the reduced palatability of extracts from damaged individuals of *D. dichotoma.*

The dominant *Dictyota* diterpenes, such as pachydictyol A, dictyol E, dictyol B acetate, and dictyodial, which are well known to function in defence of *Dictyota* against herbivores (Hay et al., 1987; Cronin and Hay, 1996a; Cruz-Rivera and Hay, 2003), appear to belong to the constitutive, rather than activated, defences of *Dictyota spp.*. Moreover, in a comprehensive profiling involving GC/MS and HPLC/MS of extracts (Fig. 1) and derivatized samples (silylated, methylated, PFBHA treatments, data not shown) from intact and wounded algae no significant changes of the metabolic profiles were detected upon tissue disruption.

In contrast, significant changes in gaseous volatiles could be detected after mechanical tissue disruption that should simulate the grinding action of herbivores feeding on *D. dichotoma* (Cetrulo and Hay, 2000). Solid phase micro extraction (SPME) coupled with GC/MS identified trimethylamine (TMA) and dimethylsulphide (DMS) as major metabolites released upon disruption of *Dictyota* tissues. In contrast, these volatiles were undetectable, or detectable in only trace amounts, from intact individuals of *D. dichotoma* (Fig. 2).

We developed a SPME / GC / MS based method to simultaneously quantify TMA and DMS in wounded algae. SPME is a fast and efficient technique for extracting organic volatiles from a variety of matrices (Li et al., 2004; Mills et al., 1999; Namiesnik et al., 2003). The use of SPME for the extraction of TMA has previously been demonstrated for the investigation of urine samples (Mills et al., 1999) and was adapted for freshwater samples (Pohnert and von Elert, 2000). Protocols are also available for the extraction of DMS using this method (Niki et al., 2004; Yassaa et al., 2006). Optimization of experimental conditions showed that exposure of a CAR / PDMS fibre to TMA and DMS in head space vapour over 200 mg *Dictyota spp.* in a 4 ml GC vial for 20 min at room temperature gave the best results. Volatile substances from the sample matrix are adsorbed onto this fibre and subsequently desorbed into the injector of a gas chromatograph / mass spectrometer. Mass spectrometric detection provides a reliable quantification even without base line separation of DMS and TMA.

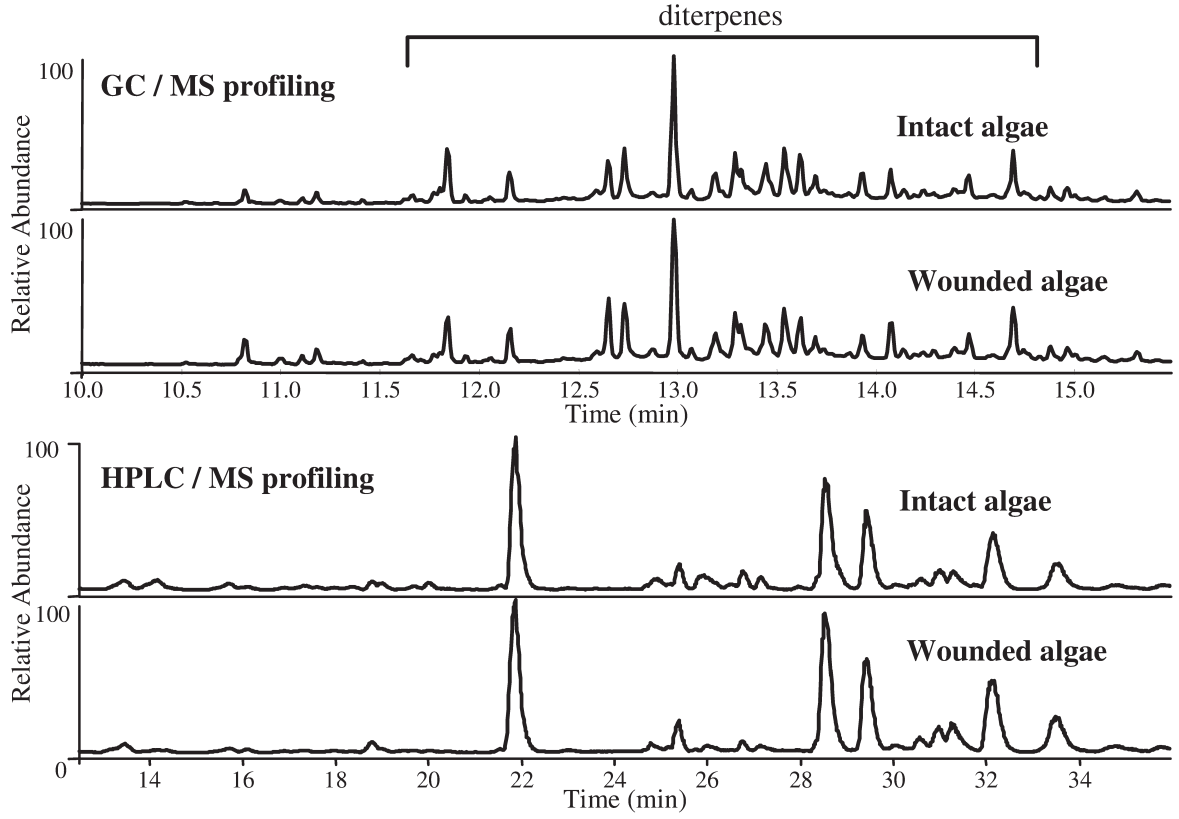
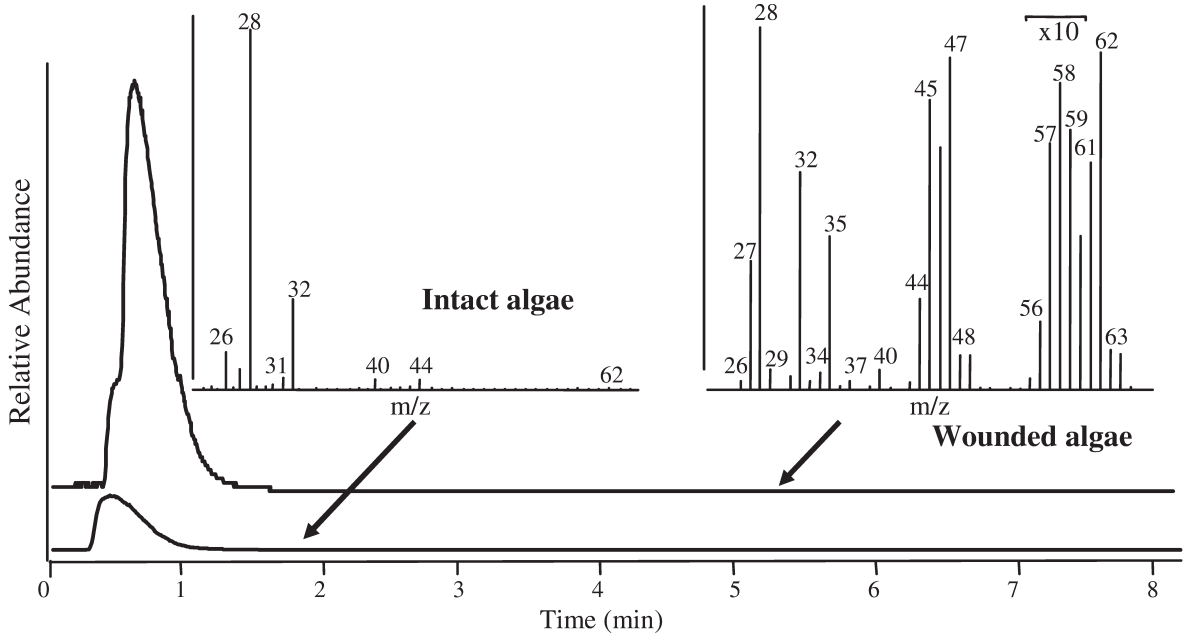


Figure 1. Top: Metabolic GC/MS profiling of dichloromethane extracts obtained from intact and mechanically wounded (5 min) pieces of *Dictyota dichotoma.* Bottom: HPLC / MS profiling of methanol extracts obtained from intact and mechanically wounded (5 min) pieces of *Dictyota dichotoma.* Similar profiles were found after prolonged (up to 48 hours) incubation after tissue disruption.



TMA: calculated 59.0732 m/z, measured 59.0735 m/z; DMS: calculated 62.0189 m/z, measured 62.0190 m/z

Figure 2. GC / MS of SPME samples from intact and mechanically wounded *Dictyota dichotoma.* The inserts show summed-up mass spectra (0.3–1.3 min); for clarity the region beween 56 and 50 was increased factor 10.

Both volatiles were identified by MS using the molecular ion and characteristic fragment ions. Signals were verified by high resolution mass measurements (TMA: calculated 59.0732 *m/z*, measured 59.0735 *m/z*; DMS: calculated 62.0189 *m/z*, measured 62.0190 *m/z*), which allow detection without interfering signals from other sources. Calibration was carried out at three different concentrations using ion trace chromatograms of *m/z* 59 for TMA and of *m/z* 62 for DMS. The analysis was linear for TMA in artificial medium over the range of 1 to 38.6 μg ml^{-1} with r^2 values between 0.78 and 0.98. For DMS quantification was possible over a concentration range of 0.5 to 12.5 μg ml^{-1} medium; r^2 values ranged from 0.84 to 0.99. This linear range makes the method suitable for the quantification of TMA and DMS in algal samples. Ageing of SPME fibres as well as ageing and contamination of the GC injector and column resulted in significant variability between the different sampling campaigns carried out over several months. This required pre-calibration before each sampling campaign. Quantification yielded up to 50 μg DMS g^{-1} *D. dichotoma* and up to 170 μg TMA g^{-1} *D. dichotoma* after mechanical tissue disruption (Table 1). While quantification of volatiles from vortexed algal material can be done reliably using this method, we cannot suggest any accuracy concerning the true values encountered by the feeding herbivores. In this situation speed and degree of tissue damage as well as local gradients might lead to significantly different effective concentrations.

A high variability in DMS release following mechanical damage was observed during different sampling campaigns. In a first sampling late spring, relatively small amounts of DMS (up to 10 μg g^{-1} alga) were detected, while in late autumn, *D. dichotoma* released up to 50 μg DMS g^{-1} alga. These results apply to freshly collected algae (data not shown) as well as to those cultured over several days under standard conditions (Table 1). During our study we did not monitor DMS production during an entire annual circle so it cannot be concluded if this observed variability is due to seasonal variations, as it is observed for other brown algal metabolites (e.g. Arnold and Targett, 1998; Hellio et al., 2004).

Simple volatile amines as well as DMS have been reported previously from marine macroalgae (Hartmann and Auferman, 1973; Herrmann and Jüttner, 1977; Steiner and Hartmann, 1968; Steinke et al., 2002b). Several surveys on DMSP-content of algae have been published using an indirect quantification of DMS released upon base treatment. Generally brown algae were found to produce little or no DMSP depending on the detection limit in the respective studies (see e.g. Reed, 1983; Karsten et al., 1990; Bischoff et al., 1994). In accordance with these studies, *D. dichotoma* used in our experiment released low concentrations of DMS after wounding (up to 50 μg DMS g^{-1}; Table 1). This low value is due to the low DMSP concentrations around 72 μg g^{-1} found in intact *D. dichotoma* (Wiesemeier and Pohnert, 2007). In comparison green algae such as *U. intestinalis* harbour significantly elevated DMSP concentrations of 0.42–2.1 mg DMSP g^{-1} (Reed, 1983). Reed (1983) attributed the low detected DMSP levels in brown algae to contamination by epiphytes such as diatoms. To test whether this holds true for *D. dichotoma* we investigated axenic cultures of this alga. Since increased DMS and TMA levels were also found in these cultures after tissue disruption we could show that at least this brown alga is itself capable to release DMS after wounding.

Table 1. Quantification of trimethylamine and dimethylsulphide in the headspace over mechanically damaged and intact *Dictyota dichotoma* from the Atlantic coast of Roscoff, France (n=10 in all cases).

	Wounded algae μg / g algae	Intact algae μg / g algae
Trimethylamine (TMA)		
June 2005[a]	70–170	< 50
November 2003[b]	80–150	< 50
Dimethylsulphide (DMS)		
June 2005[a]	5–10	< 1.5
November 2003[b]	30–50	< 1.5

[a] kept in culture for 3–5 weeks
[b] kept in culture for 1–2 weeks

DMS release in elevated concentrations as found in *Ulva* spp. can function as a feeding inhibitor for sea urchins (Van Alstyne and Houser, 2003; Van Alstyne et al., 2001). But no inhibition at the rather low DMS concentrations we found for damaged *D. dichotoma* were observed. A role for trimethylamine in the chemical defenses of macroalgae has not been demonstrated as well.

We produced artificial, algal based foods with and without volatiles to directly assess the effects of TMA, DMS, and the biosynthetically connected acrylate released after tissue disruption of *D. dichotoma.* The amphipod *Amphithoe longimana* used in our bioassays is widely distributed along the Atlantic coast of North America and can be closely monitored under controlled conditions in the lab. *A. longimana* consumes the brown alga *Dictyota menstrualis* (Hay et al., 1987; Duffy and Hay, 1991; 1994; Cruz-Rivera and Hay, 2003), which is closely related to *D. dichotoma.* This relationship is demonstrated by their similar secondary chemistry. Interestingly, wound activated DMS and TMA release was also observed in the related alga *Dictyota menstrualis* collected in Morehead City, North Carolina, USA. Even if the amount

of volatiles could not be quantified after transport from the USA to the lab in Switzerland (data not shown), this observation indicated that the closely related Dictyota species which co-occurs in the same habitat as the herbivore *A. longimana* that was used in our bioassays shows a similar wound activated metabolic reaction as *D. dichotoma.* The dictyols produced by *D. menstrualis* minimally affect feeding by this amphipod but strongly deter feeding by fishes and sea urchins (Hay et al., 1987). Since these amphipods are obviously affected by defence compounds other than the dominant terpenes they represent excellent model organisms for the investigation of defence properties of the identified volatiles (Cruz-Rivera and Hay, 2003).

Performing bioassays on the identified compounds poses certain problems because of the volatility, and thus rapid diffusion and loss, of the metabolites of interest. Thus, accurately predicting the local concentrations of volatile metabolites released upon tissue disruption, and the concentrations experienced by the feeding herbivore is a major challenge (Pohnert, 2004). Prediction of the actual concentration of defence metabolites during herbivore feeding is further complicated by differences in secondary metabolite concentrations between algae growing in different habitats as well as algae separated by only a few meters (Hay and Steinberg, 1992; Cronin and Hay, 1996b; Sotka et al., 2003; Teixeira et al., 1990; Teixeira et al., 2001; Taylor et al., 2003; Vallim et al., 2005). In addition, the content of secondary metabolites can also vary within one alga (Cronin and Hay, 1996a; Cronin and Hay, 1996b; Pavia et al., 2002; Taylor et al., 2003) and thus only estimated amounts of defence metabolites can be used in bioassays. For the starting concentrations in our bioassays we used the DMS and TMA concentrations detected within a 20 min period after tissue disruption of the alga. Similar concentrations of non volatile acrylate were used as well. To compensate for the above mentioned insecurities associated with tissue wounding, assay design and for the potentially different local concentrations that might be experienced by a feeding herbivore, further choice assays with elevated starting concentrations corresponding to 10x and 100x of the detected levels were performed.

Amphipods are small and feed slowly, but volatiles begin escaping from the foods as soon as they are removed from the sealed tubes. Thus our bioassay could run for only 25 min, and, despite the fact that feeding activity was observed, during this time period we were not able to measure the amount of food consumed (see also Cruz-Rivera and Hay, 2000; 2003). The behavioural association assay used here is a surrogate for monitoring of feeding because these amphipods tend to live on, and thus associate spatially with, the foods that they consume (see also Cruz-Rivera and Hay, 2000; 2003). To statistically evaluate food choice, we used standard deviation and Wilcoxon paired–sample tests. To assess the loss of volatiles during the preparation of the artificial food and during the assay volatiles in the treated agar pieces were quantified: $36.4 \pm 5.7\%$ (n=3) of the initially added TMA and $10.6 \pm 0.4\%$ (n=3) of DMS were found after food preparation. Additional losses due to diffusion into the water during the assay result in levels of $8.2 \pm 1.7\%$ (n=3) of the initially used TMA and $4.2 \pm 1\%$ (n=3) of DMS after the 25 min assay. Taking these values into account, assays were run with TMA levels declining from 109.2 $\mu g\ g^{-1}$ to 24 $\mu g\ g^{-1}$ during the assay period and with DMS levels from 4.2 $\mu g\ g^{-1}$ to 1.7 $\mu g\ g^{-1}$. These values are in the lower range of those found in *D. dichotoma* (Table 1). Additionally 10x and 100x these concentrations were used. Amphipod preference for associating with the control diet or the equivalent diet containing some of the volatiles (treatment diet) was noted every five minutes for twenty five minutes after adding amphipods into vessels. At values around the lowest concentrations found in wounded algae, DMS alone did not significantly deter amphipods (Fig. 3 top panel). When increased to 10x of this concentration a non-significant feeding inhibition was observed, if applied as a mixture of DMS and acrylic acid, a non significant stimulation was observed (Fig. 3). Only at the 100x concentration, DMS significantly deterred amphipods (data not shown), these elevated active concentrations, which are not released by the alga investigated in this study, correspond to those required for the inhibition of sea urchin feeding (Van Alstyne and Houser, 2003). Further TMA itself, acrylate and binary mixtures of these compounds had no defensive properties at the lower concentrations found in wounded algae and at 10x elevated concentrations TMA resulted in reduced food preference, while acrylic acid made the food particles more attractive (Fig. 3). Thus, natural levels of each compound alone did not significantly deter this amphipod.

However, when an alga is wounded, it will not release individual pure compounds but a complex mixture; it will be the effect of this mixture that is critical in determining whether herbivores continue to consume the alga or reject it. Mixtures of DMS and the biosynthetically connected acrylate as well as mixtures of DMS, acrylate and TMA were thus tested in bioassays against amphipods. Interestingly, synergistic effects occurred and the mixture of synthetic compounds resembling the natural blend of volatiles did significantly deter amphipods at concentrations detected in *D. dichotoma.* If given a choice between a

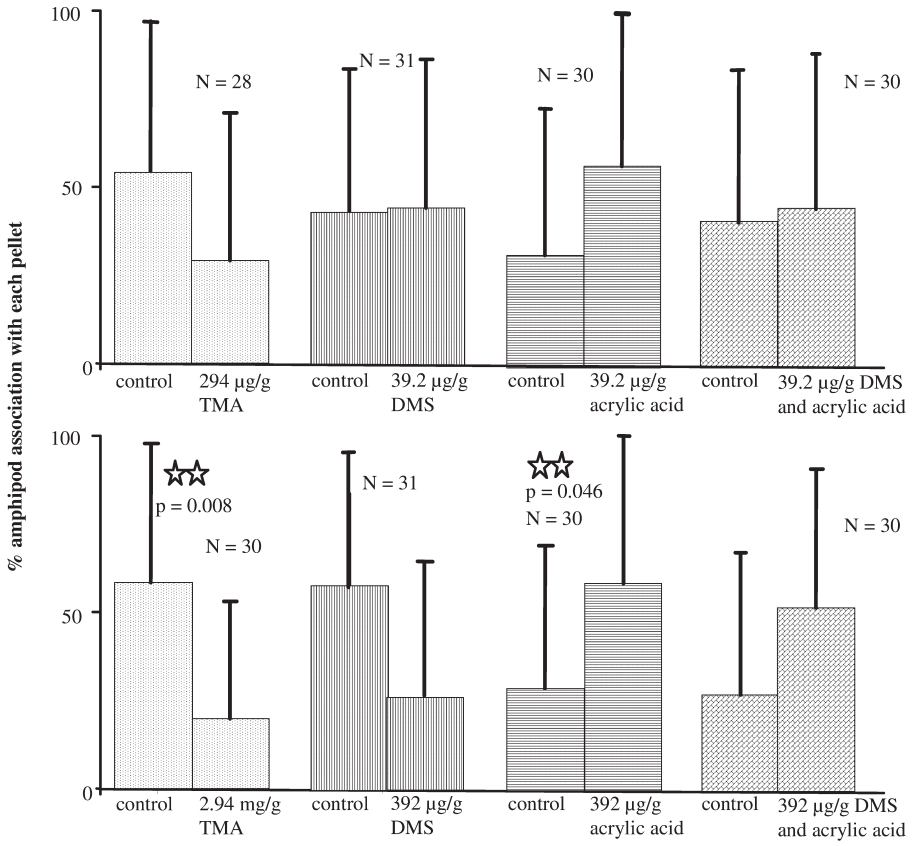


Figure 3. Amphipod food preference assays for individual compounds and a mix of dimethylsulphide and acrylate (error bars = standard deviation). The given values for DMS, TMA and acrylic acid correspond to those used during food preparation. The actual values during the assays are significantly lower (see results and discussion). None of the detected differences for the lower concentrations is significant (top panel, Wilcoxon paired–sample tests). Besides with TMA no significant feeding inhibition was observed at 10x of this concentration, acrylic acid alone stimulated feeding (bottom panel, Wilcoxon paired–sample tests).

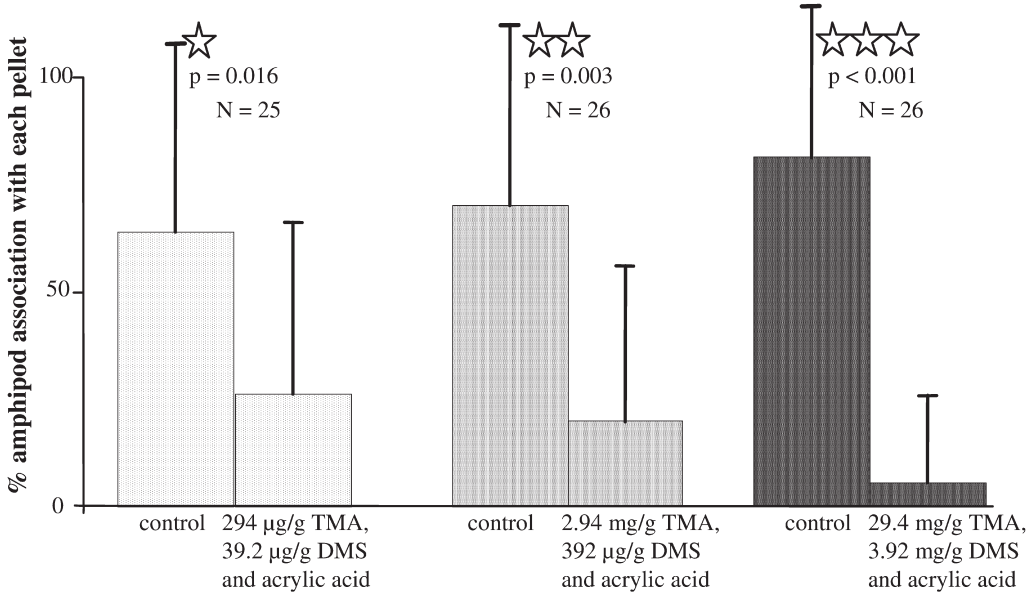


Figure 4. Amphipod food preference assays for mixed components (error bars = standard deviation, N=number of replicates, p values and stars are derived from Wilcoxon paired–sample tests, all differences were significant). The given values for DMS, TMA and acrylic acid correspond to those used during food preparation. The actual values during the assays are significantly lower (see results and discussion).

sample of artificial food with and without added volatiles, the amphipods significantly preferred the control food. This synergistic effect was also observed at elevated concentrations (Fig. 4). Thus, the mixture of the volatiles released upon tissue disruption constitutes an activated defence of *D. dichotoma.* Evidently, the herbivores are able to detect a volatile blend and avoid food sources releasing these volatiles. Blend recognition of aquatic herbivores is involved in food selection (Höckelmann et al., 2004) and mixtures of chemical defensive compounds, or mixes of chemical and structural defences, have been proven to be more active than the single component (Berenbaum and Neal, 1985; Hay et al., 1994). Here we show for the first time that this also holds true for gases released upon brown algal cell disintegration. Interestingly, when offered a choice between *D. menstrualis* and *Ulva intestinalis* under lab conditions, *A. longimana* strongly preferred *U. intestinalis* (Cruz-Rivera and Hay, 2003) despite the fact that this alga contains significantly more DMSP than the brown alga (Reed, 1983). This indicates that not the DMSP content but the activated release of a blend of volatiles determines the defensive potential of these metabolites.

The activity of the mixture tested in this assay might motivate further work on the effect of mixtures of DMS, acrylic acid and DMSP. Thus, e.g., Strom et al. (2003) showed that DMSP, but not DMS or acrylate alone inhibited protist grazing on *Emiliania huxleyi.* This refuted an earlier hypothesis of Wolfe et al. (1997). However, Strom et al., did not test mixtures of DMS and acrylate, which might result in different effects.

Conclusion

Using SPME and GC/MS, we could show that *D. dichotoma* releases DMS and elevated amounts of TMA after wounding. A blend of these volatiles and acrylate contributes to the activated chemical defence of this alga.

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